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MINIREVIEW

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Pharmacology and physiological function of the orphan GPRC6A receptor

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Abstract

The G protein-coupled receptor GPRC6A (GPCR, Class C, group 6, subtype A) is a $G_{q/11}$-coupled receptor widely expressed in human and rodent tissues. The proposed endogenous ligands are L-amino acids, divalent cations, osteocalcin and testosterone. This MiniReview provides an updated overview of the literature including the latest in vitro and in vivo studies. GPRC6A forms homodimers, it undergoes constitutive internalization, and very interestingly, the reason for the intracellular retention of the human receptor has been revealed. Multiple physiological functions of GPRC6A have been suggested based on studies using three different global GPRC6A knockout (KO) mouse models where exon II, exon VI or the full locus has been deleted. The newest studies on the full locus GPRC6A KO model show intact glucose and bone homoeostasis with a minor reduction in serum osteocalcin levels. Unfortunately, the physiological function of the receptor remains elusive due to a general lack of consensus/validation of reported phenotypes of the different KO models, and more research is thus warranted to uncover the physiological function. Recent discoveries of human genetic variants that cause either a premature stop codon or an intracellular retention of the receptor point towards human population studies as the preferred approach to continue studies on the function of GPRC6A.

1 | MOLECULAR FUNCTION AND PHARMACOLOGY OF THE GPRC6A RECEPTOR

In 2004, the class C of G protein-coupled receptors (GPCRs) expanded with an additional member, as a new GPCR termed GPRC6A (GPCR, Class C, group 6, subtype A), was cloned from a human kidney complementary deoxyribonucleic acid library. Like the other canonical class C receptors, GPRC6A is characterized by having an unusual long amino-terminal domain (590 amino acids), which includes a Venus flytrap domain and a cysteine-rich domain containing nine conserved cysteine residues, and a 7-transmembrane (7TM) domain containing three extra- and three intracellular loops and an intracellular C-terminal (Figure 1).[1] The receptor forms homodimers through a large hydrophobic interface and a disulphide bridge in the Venus flytrap domain, and the receptor is N-glycosylated at seven extracellular asparagine residues, which modulates surface expression and function.[2] The amino acids are highlighted in the snakeplot in Figure 1. The receptor is constitutively internalized and suggested to be recycled via the Rab11-slow recycling pathway to ensure a steady pool of receptors in the plasma membrane despite a chronic exposure to the omnipresent L-amino acids and divalent cation agonists.[3]

Parts of the present MiniReview have been published previously in the PhD thesis of Dr Christinna V. Jørgensen.

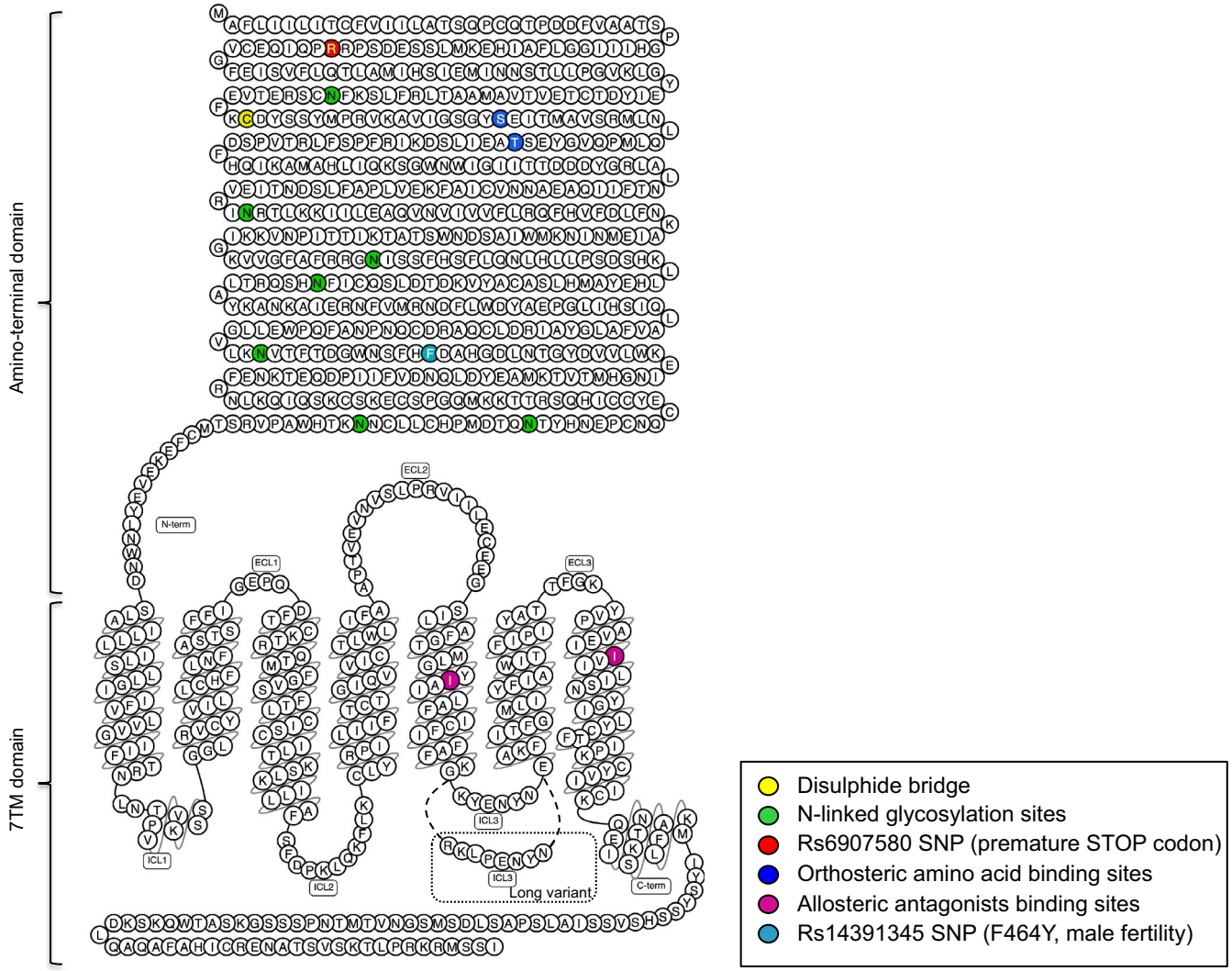

FIGURE 1 Snakeplot representation of structural features of human GPRC6A. The cysteine residue involved in homodimerization and the N-linked glycosylation sites are shown in yellow and green, respectively.[2] Residues important for binding of an orthosteric ligand are shown in blue,[5] while purple residues are involved in binding of allosteric antagonists.[11,45] The long variant of the receptor (rs386705086) is indicated in the dashed area in the ICL3 region, and the premature STOP codon (rs6907580) that is partially linked to the long variant is shown in red.[7] The SNP reported to be associated with male infertility (rs14391345) is shown in light blue.[74]

1.1 | The *GPRC6A* gene

The *GPRC6A* gene contains six exons, and a total of three splice variants are produced, with the longest isoform, isoform 1 being the most abundant.[1] The human ortholog of the isoform 1 protein is 926 amino acids long and shares 34% sequence identity with the calcium-sensing receptor (CaSR), 28% with the taste receptor (T1R1) and 24% with metabotropic glutamate receptor subtype 1 (mGluR1). The mouse and rat GPRC6A sequences share 93% identity, while the mouse homolog is only 80% identical to the human version of the receptor.[4-6] A main difference between the human ortholog and the murine version is that the most common genetic variant of the human GPRC6A receptor is not expressed at the cell surface in recombinant systems.[1,5] The reason for the intracellular retention took more than a decade to reveal, and it was not until 2017 that the answer was found. An insertion/deletion variation in the third intracellular loop in human GPRC6A was identified, which is responsible for the intracellular retention of the receptor. The insertion/deletion variation results in a two amino acid shorter version of the loop, which changes from FAFKG**KY**ENY (wild-type (WT)) to FAFKG**RKLP**ENY (rs386705086) (See Figure 1). The short version is only found in humans and not even in the 99% identical bonobo chimpanzee[7] or 16 other species.[8] The short version of the receptor is by far the most prevalent in humans with a genotype frequency ranging from 60% in the African population to 99% in the East Asian population. The longer, cell surface-expressed variant of GPRC6A is most prevalent in Africa. In European and Asian populations, the long variant is partly linked to a premature stop codon at amino acid

position 57 (rs6907580, shown in red in Figure 1) resulting in a truncated non-functional receptor.[7]

1.2 | Expression pattern

The expression pattern of a given receptor can often give indications to the physiological function of the receptor. In case of GPRC6A, the messenger ribonucleic acid (mRNA) expression levels have been analysed in multiple tissues and species with reverse transcription polymerase chain reaction and in situ hybridization. In general, all studies show broad but low levels of expression. Tissues and organs with GPRC6A expression include brain, lung, liver, heart, kidney, pancreas, testis, skeletal muscle, bone and fat (for overview, see Clemmensen et al[9]). To date, there are no validated GPRC6A antibodies to study expression at the protein level, which would have been a valuable tool to investigate the functional tissue expression.

1.3 | Ligands of GPRC6A and intracellular signalling

Quickly after the first cloning of GPRC6A, the receptor was deorphanized by us and the David Hampson group.[4,5] Several other groups have subsequently confirmed that recombinantly expressed GPRC6A is a promiscuous L-α-amino acid receptor most potently activated by the positively charged L-arginine, L-lysine and L-ornithine (micromolar range).[4,5,10-12] GPRC6A is able to sense amino acid concentrations within a physiologically relevant range in mice.[13] Divalent cations have been shown to act as positive allosteric modulators in some studies, whereas other studies have shown agonistic activity of Ca^{2+} putatively caused by ambient L-amino acids levels.[1,4,5,13-16] These two classes of ligands fit very well with the general characteristics of class C GPCR ligands.[17,18]

The bone-derived peptide osteocalcin has also been proposed to act as a GPRC6A agonist. Several in vitro studies have shown that the effects of osteocalcin are dependent on GPRC6A using transfected cells or GPRC6A small interfering RNA.[14,19-21] However, we and other investigators have not been able to confirm any agonistic effects of osteocalcin on GPRC6A in vitro.[10,15,22] The cause of these discrepancies is at present unclear but could be speculated to be due to the use of different sources of osteocalcin, which is known to exist in different forms/conformations, "stickiness" of the ligands to plasticware, indirect actions of the ligands via other targets present in some but not all expression systems or use of different cell expression systems, signalling end-points and/or signalling assays leading to differences in assay sensitivity. More studies are needed to fully elucidate this.

Osteocalcin is exclusively synthesized in osteoblasts, where it undergoes post-translational γ-carboxylation at three glutamate residues. The carboxylation makes osteocalcin capable of binding Ca^{2+}, adopting an α-helical structure and thereby binding to hydroxyapatite, the mineral component of bone.[23,24] It constitutes the most abundant non-collagen protein in bones. During bone resorption, osteocalcin is decarboxylated, which decreases its affinity for bone hydroxyapatite, and thereby facilitates its release into the circulation.[25,26] A number of metabolic and endocrine functions have been reported for uncarboxylated osteocalcin, where the groups of Quarles and Karsenty suggest these are mediated through the activation of GPRC6A.[14,19,21,27,28]

One argument that has been used to state that GPRC6A is an osteocalcin receptor in vivo is that the exon II GPRC6A knockout (KO) mouse model is a phenocopy of osteocalcin KO mice.[29] This is challenged by a recent study characterizing a rat osteocalcin KO model, where the osteocalcin-deficient rats do not display the same metabolic disturbances as the mice.[8,30-32] This raises the question if the function of osteocalcin is conserved across species or if the metabolic effects in the mouse model arise from off-target events since the adiponectin receptor progestin and adipoQ receptor family member VI were also deleted when generating the osteocalcin-deficient mice.[30,31] Additional/alternative osteocalcin receptors may thus exist. The orphan GPR158 has been proposed to mediate the effects of osteocalcin in the brain, where osteocalcin has been reported to act in a GPRC6A-independent manner.[33,34] With the currently available data, it is hard to draw a conclusion on the agonistic activity of osteocalcin on the GPRC6A receptor, and more experiments in vitro and in vivo are therefore warranted.

The steroid hormone testosterone has also been suggested as an endogenous GPRC6A ligand by the Quarles group.[35,36] Another group proposed that it is not testosterone but the sex hormone binding globulin that activates GPRC6A.[37] As with osteocalcin, we and other investigators have not been able to reproduce the agonistic activity of testosterone on GPRC6A.[15,38] Hence, the receptors responsible for the effects of osteocalcin and the non-genomic effects of steroids have not yet been fully elucidated. In addition to osteocalcin and testosterone, gallates in green tea and angiotensin have also been reported as GPRC6A agonists.[39,40] In general, these proposed ligands are structurally very different to the classic agonists of class C receptors, which are thought to have developed through evolution as amino acid- and cation-sensing receptors.[17] In silico docking of L-arginine indicated a tight fit within the orthosteric binding pocket in the Venus flytrap domain of GPRC6A with no additional space for larger ligands,[41] which is supported by the observation that mutations of S149A or S172A in the orthosteric binding site eliminated L-lysine and L-arginine agonist activity (Figure 1).[5] It is presently unclear where the Ca^{2+} binding site(s) are located,

but recent X-ray crystal structures of the extracellular amino-terminal domain of the most closely related receptor, CaSR, have identified a handful of putative cation binding sites which might be preserved in GPRC6A.[42,43] Although osteocalcin and testosterone have been proposed to bind outside the orthosteric site,[21,36,37] more studies are warranted to confirm or reject if these are true GPRC6A ligands given the previously mentioned negative studies. In conclusion, there is wide consensus that GPRC6A is activated by proteinogenic L-amino acids and modulated/activated by Ca^{2+} and other cations, while there is conflicting evidence for the other proposed ligands.

The putative endogenous ligands all have multiple targets in animals, which unfortunately limit their use as pharmacological tool compounds. The development of potent and selective agonists and antagonists would be valuable in enabling the identification of the physiological function of GPRC6A and for the evaluation of the receptor as a potential therapeutic target. To date, we have screened L-amino acid analogues, where several were equipotent with the natural basic amino acids but did not possess sufficient selectivity.[44] With respect to antagonists, it has been shown that the CaSR allosteric modulators calindol and NPS2143 can antagonize GPRC6A with inhibitory activity in the micromolar range, which limits their usability given their higher potency on CaSR.[11] Via a chemogenomic approach using a 2-phenyl-indole scaffold, we identified three negative allosteric modulators of GPRC6A including "Compound 3."[45] Potency and selectivity were further improved with an analogue screen, making "Compound 7" and "Compound 34b" the most potent and selective allosteric modulators to date.[46] Calindol and "Compound 3" bind in the 7TM domain as shown in Figure 1, which fits with observations on other class C GPCRs where allosteric modulators also bind the transmembrane helices.[18] Unfortunately, the compounds available today still need further optimization of selectivity, potency and solubility before they can used in in vivo studies.

In line with the array of multiple proposed ligands, several signalling pathways have been suggested downstream of the GPRC6A receptor. In our hands, GPRC6A specifically activates the $G_{q/11}$ signalling pathway, which has been shown in human embryonic kidney 293 (HEK293) cells transiently transfected with human/mouse/rat GPCR6A, a stably expressing mouse GPRC6A-Chinese hamster ovary cell line and in *Xenopus* oocytes expressing mouse GPRC6A.[5,7,11,13,15] The $G_{q/11}$ pathway signalling has been confirmed by other groups.[4,10] In addition to $G_{q/11}$ coupling, other groups have reported signalling from mouse GPRC6A-transfected HEK293 cells via the G_s, $G_{i/o}$ and ERK1/2 signalling pathways of which the latter is plausible downstream of $G_{q/11}$ signalling.[14,35,36,47] However, we have been unable to confirm the G_s or $G_{i/o}$ signalling,[15] which could be due to differences in receptor/G protein expression levels and/or assay sensitivity.

2 | SUGGESTED PHYSIOLOGICAL FUNCTIONS OF GPRC6A

The combination of the wide expression pattern and the fact that GPRC6A is activated by a broad spectrum of omnipresent L-amino acids led to the hypothesis of GPRC6A acting as an amino acid sensor that regulates metabolism and growth.[48] As detailed later, GPRC6A has also been suggested to be involved in bone homoeostasis, male fertility, inflammation and prostate cancer. Due to lack of GPRC6A ligands with sufficient potency and selectivity, the (patho)physiological functions of the receptor have mainly been studied by phenotyping GPRC6A KO mice.

To date, three global GPRC6A KO models have been published. In 2008, the group of Quarles published the first GPRC6A KO model, where exon II, which encodes a minor part of the Venus flytrap domain, had been deleted.[49] The following year, the group of Bräuner-Osborne generated the second global GPRC6A KO model, where exon VI was disrupted. This exon encodes the entire 7TM domain and carboxy-terminal of the receptor.[50] Lastly, the Knockout Mouse Project (KOMP) has made a full locus GPRC6A KO model available, where the entire *Gprc6a* gene has been replaced by the *lacZ* gene. Despite the availability of genetically modified animals, no consensus about the physiological function(s) of GPRC6A has been obtained, but multiple physiological functions have been proposed and tested in the three KO models as detailed below.

2.1 | GPRC6A and glucose metabolism

So far, most studies have focused on the potential function of GPRC6A in regulating glucose metabolism.[47,49,51-53] Despite several studies within the field, it remains uncertain if GPRC6A holds a function in various aspects of metabolism. The lack of consensus could have arisen due to the use of different KO models, or due to differences in the animal facility environments or microbiome, which are known to affect metabolism studies.[54] We have also noted that some studies have used relatively small animal groups considering the number of biomarkers investigated, which could lead to underpowered studies and false positives (type I errors).[55] The GPRC6A exon II KO mice displayed hepatic steatosis, increased fasting serum glucose levels, decreased insulin levels and were insulin-tolerant. In addition, they had reduced lean body mass and increased body fat mass.[49] Our exon VI KO mice exhibited normal bodyweight development up to an age of 38 weeks, indicating that the regulation of energy balance was intact in GPRC6A exon VI KO mice fed a standard chow diet.[50] They did not suffer from increased body fat mass, insulin resistance or glucose intolerance in contrast to the exon II model.[51] The

full locus GPRC6A KO mouse has been reported to have normal basal glucose levels, normal response to a glucose tolerance test, no differences in insulin sensitivity or body composition, which is in accordance with the phenotype of the exon VI KO model.[53,56] Additionally, the full locus GPRC6A KO mice displayed no susceptibility differences in developing dysmetabolism compared to WT in response to a high-dose glucocorticoid treatment.[56] In 2011, Pi et al published that GPRC6A mediated effects of osteocalcin in vivo. An intraperitoneal injection of osteocalcin induced insulin secretion in WT mice but not in GPRC6A exon II KO mice. Interestingly, the basal serum insulin levels in this study were similar between genotypes, which stand in contrast to the earlier finding during the initial phenotyping.[19,49]

The GPRC6A agonist L-arginine is a potent inducer of insulin secretion.[57] Based on the fact that GPRC6A is expressed in the endocrine pancreas, it was an obvious hypothesis to test whether L-arginine acts through GPRC6A to stimulate insulin release.[58] L-arginine potently induced insulin secretion in vivo and ex vivo equally well in both WT and GPRC6A exon VI KO mice.[51] Pi et al tested the same hypothesis in isolated islets from their exon II KO mice. In contrast, they observed reduced insulin levels and stimulation index in response to L-arginine in GPRC6A-deficient islets compared to WT. In this study, they confirmed their initial finding of decreased basal serum insulin levels in GPRC6A-deficient mice.[47] Taken together, these data cannot support or reject the hypothesis of GPRC6A being involved in L-arginine-induced insulin secretion. Additional studies are required to conclude whether or not GPRC6A has a direct role in L-arginine- and/or osteocalcin-induced insulin release.

Moreover, we have used exome, biomarker and register data of 6000 Danes from the Inter99 population sample cohort (CT00289237, ClinicalTrials.gov)[59] to compare human carriers of the functional surface-expressed long ICL3 variant of GPRC6A and the non-functional stop codon KO variant with the intracellular retained short ICL3 WT variant (frequencies in the Danish population: 0.003, 0.068 and 0.93, respectively). The vast majority of metabolic parameters were not significantly different between the compared genetic groups, but with the caveat of having very small non-WT groups, it was shown that male carriers of the functional long ICL3 variant displayed an increased 30-minutes insulin response in an oral glucose tolerance test and female KOs displayed increased 2-hour glucose levels compared to WT with short ICL3.[7] Although these results are based on a small sample size in the non-WT groups and interpretation requires caution, the positive findings align with the mouse phenotypes showing impaired metabolism in mouse KOs and thus support a role, albeit minor, of GPRC6A in metabolism.

In another genetic study on a cohort of 392 adults of both genders, Di Nisio et al[60] have linked the very common Pro91Ser genotype (rs2274911, 72% frequency) with increased fasting insulin and HOMA-IR. Unfortunately, it was not investigated if the patients were carriers of the long or short ICL3 variant and/or the Arg57STOP variant.

2.2 | GPRC6A and energy homoeostasis

Our GPRC6A exon VI KO mice displayed normal body-weight gain under standard physiological conditions.[50,61] However, if we challenged the mice with a high-fat diet for more than 25 weeks, it induced hyperphagia in the KO animals and consequently obesity with disturbances in metabolic parameters.[52] It should be noted that the difference between WT and KO was initiated when the mice were separated into single metabolic cages halfway through the study, whereby stress might be an additional factor in the observed metabolic phenotype. The Murphy group demonstrated that GPRC6A was not required for the effects of low- or high-protein diets on energy homoeostasis. The full locus KO mice displayed normal food intake and body-weight when compared to WTs after 9 and 35 days.[53] It remains unknown why the GPRC6A KO mice are more susceptible to high-fat diet-induced obesity, while they display normal responses to high-protein diets, but as mentioned above stress from single housing in a new environment could be a trigger. Moreover, the relatively short exposure periods in the protein diet studies or compensatory mechanisms may be part of the answer. In relation to energy homoeostasis, our group has published an exercise phenotype for GPRC6A exon VI KO mice. The exon VI KO mice ran 50% more than WT mice if introduced to voluntary running in a wheel, while there was no difference in forced maximum running speed (cm/s).[61] Interestingly, Mera et al (2016) reported that GPRC6A exon II KO mice ran significantly shorter distances than WT mice during a forced endurance test until exhaustion.[28] The possible mechanisms behind these exercise phenotypes remain unexplored. We can only speculate if the altered exercise behaviour and the susceptibility to diet-induced obesity/stress responses are interrelated.[9] Given the observatory nature of the studies, it is hard to conclude what exact function GPRC6A holds, but the studies point towards a role of the receptor in some aspects of energy homoeostasis including exercise behaviour.

2.3 | GPRC6A as a gastrointestinal nutrient-sensing receptor

Given that GPRC6A is a promiscuous amino acid sensor and is expressed in our digestive system, the receptor has

been proposed as a candidate to sense digested amino acids in the gastrointestinal tract.[62,63] Two groups have shown that GPRC6A is involved in L-ornithine-induced glucagon-like peptide-1 (GLP-1) release in the intestinal L cell line GLUTag.[10,64] However, Oya et al were not able to measure L-ornithine-induced GLP-1 release from mixed primary cultures of the mouse small intestine.[64] Alamshah et al[65] revealed that L-arginine stimulated peptide YY secretion from both WT and GPRC6A KO primary colonic L-cells, whereas the L-arginine-mediated GLP-1 secretion was attenuated in the KO cells. To test whether this translates to the in vivo state, we have administered L-ornithine and L-arginine orally to the full locus and exon VI GPRC6A KO mouse models. We observed robust GLP-1 release, but while the GLP-1 secretion was attenuated at later time-points, there were no overall differences between KOs and WTs, demonstrating that functional GPRC6A is not required for robust secretion of GLP-1 in vivo by basic L-amino acids.[66] This suggests that other receptors or mechanisms than GPRC6A are mediating at least some of the effects of L-arginine and L-ornithine in vivo. Obvious candidate receptors include the other amino acid-sensing receptors belonging to class C GPCRs such as CaSR and T1R1-T1R3, which show overlapping tissue expression and L-amino acid preferences with GPRC6A.[67,68] In fact, CaSR has recently been demonstrated to mediate the effects of L-phenylalanine on gut hormone release, which proves that this receptor is capable of inducing GLP-1 secretion.[69]

2.4 | The role of GPRC6A in bones

Broad-spectrum amino acid-sensing receptors are thought to play a vital role in bone health.[70] L-arginine has impact on osteoblastic differentiation and osteogenesis,[70-72] and GPRC6A could potentially mediate these effects and in this way be involved in bone homoeostasis. The first phenotyping of the GPRC6A exon II KO model revealed a reduced bone mineral density (BMD) in the KO mice due to diminished mineralization.[49] The following year, our group performed the first characterization of the exon VI KO model. In contrast to the work of Pi et al, there was no obvious bone phenotype of this model. There were no abnormalities in the bone microstructure, BMD or alterations in biomarkers of bone turnover in 13-week-old GPRC6A exon VI KO mice compared to WT littermate controls.[50] Pi et al published a follow-up paper on their exon II KO model, where they expanded the characterization of the potential bone phenotype, which again showed reduced BMD. However, the reduction in BMD in 16-week-old KO mice was not reflected in the microstructure of the bone. Experiments on primary osteoblasts revealed that the lack of GPRC6A impaired the ability of the cells to sense amino acids and Ca^{2+}. To further strengthen their work, they found two single nucleotide polymorphisms (SNPs) (rs686708 and rs571296) in the non-coding region of the *GPRC6A* gene associated with lower spine BMD in 1000 unrelated Americans.[73] We have recently expanded the research within this area by analysing the bone microstructures in young (9-12-week-old) and aged (47-50- and 62-66-week-old) full locus GPRC6A KO mice and WT littermates. This study revealed a minor reduction in serum total osteocalcin levels as reported for the exon II KO model[49] but no differences in long bone microstructures in agreement with the exon VI KO model.[50,56] Taken together, GPRC6A does not appear to play a critical role in mouse bone physiology.

2.5 | Does GPRC6A play a role in male fertility?

In addition to a bone and metabolism phenotype, the initial phenotyping of the exon II GPRC6A KO model revealed a feminization of the male mice measured as reduced genito-anal distance, testicular size and weight, and weight of the seminal vesicle. Testosterone concentrations were lower and estradiol concentrations elevated. Moreover, the authors described that KO-KO breeding pairs had litters roughly half the size of heterozygous (HZ)-HZ and KO-WT breeders.[49] A few years later, the observations were supported by a study demonstrating a direct role of GPRC6A in male physiology, as the group of Karsenty showed that GPRC6A was expressed in Leydig cells of the testes, where osteocalcin regulated testosterone secretion in a GPRC6A-dependent manner. Additionally, the $GPRC6A_{Leydig}$ exon II conditional KO mice exhibited feminization like the global exon II KO model.[27] A SNP (rs14391345) causing a Phe464Tyr mutation in the *GPRC6A* gene was suggested to be associated with infertility, as two infertile patients harboured this SNP and it prevented the receptor from being cell surface expressed (Figure 1).[74,75] Moreover, in another genetic study on a cohort of 343 adult infertile males and 71 cryptorchid newborns, De Toni et al linked homozygote carriers of the very common Pro91Ser genotype (rs2274911, 69%-83% allele frequency) with increased risk of oligozoospermia and cryptorchidism.[75] We now know that the majority of humans carry a short variant of the receptor that is not localized at the cell surface,[7] which was not genotyped in the two patients. The importance of the reported SNPs thus has to be further investigated. Moreover, as mentioned above, Pi et al reported agonistic activity of testosterone on GPRC6A in vitro and in vivo. Testosterone-stimulated signalling was seen in HEK-293 cells over-expressing GPRC6A and in WT cells and mice in contrast to GPRC6A-deficient cells and mice.[35]

In contrast to Quarles' results from exon II KO mice, we have not observed breeding difficulties in our KO models indicating that the exon II KO model in this aspect also suffers from a more severe phenotype than the exon VI and

full locus KO models. Additionally, we have analysed rough measurements of fertility in a Danish cohort, the Inter99 randomized control trial consisting of 6293 individuals, where we did not observe differences in testosterone levels or in the proportion of men who had children when comparing KOs (people carrying alleles with the premature stop codon at codon 57) with WTs (short ICL3 variant of GPRC6A).[7] This could indicate that the exon VI and full locus KO mice resemble humans better than the exon II KO mice. However, using additional cohorts including more individuals and different nationalities in future studies would strengthen the human genetic data.

2.6 | GPRC6A and prostate cancer

Genome-wide association studies (GWAS) in Asian populations have identified the GPRC6A/Regulatory factor X6 genomic region as a new prostate cancer susceptibility locus.[76-78] The same association has been replicated in a European population.[79] The group of Quarles followed up on this interesting association by crossing their exon II GPRC6A KO mice with the transgenic adenocarcinoma of the mouse prostate (TRAMP) mouse model. TRAMP mice displayed intraepithelial hyperplasia in the prostate after 30 weeks, whereas the GPRC6A null TRAMP mice appeared with normal prostate histology. In addition, the deletion of GPRC6A improved survival of the TRAMP mice compared to the TRAMP mice expressing GPRC6A.[80] The same group has implanted Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) GPRC6A-edited prostate cancer cells (the PC-3 cell line) in nude mice. Tumour size and weight were higher for controls compared to the CRISPR/Cas9-edited PC-3 cells with reduced GPRC6A expression, again indicating that loss of GPRC6A functionality can protect against prostate cancer.[81]

Taken together, these findings support a role of GPRC6A in prostate cancer development and progression, and future studies will reveal if targeting this receptor with an antagonist could be a complementary strategy to treat androgen-resistant prostate cancer.

2.7 | Inflammation

A less studied potential physiological function of GPRC6A is its involvement in inflammatory responses. C-reactive protein (CRP) is a general biomarker of inflammation, and a GWAS identified the GPRC6A locus as being associated with circulating CRP levels.[82] The group of Wagner showed that extracellular Ca^{2+} activated the inflammasome through GPRC6A and CaSR. Monocytes and macrophages from GPRC6A exon VI KO mice displayed reduced Ca^{2+}-induced secretion of the proinflammatory cytokines interleukin (IL)-1β, IL-1α and tumour necrosis factor. These observations were further strengthened by results from an in vivo model, where a footpad swelling was reduced in GPRC6A exon VI KO mice.[83] Three years later, it was reported that aluminium salts used as adjuvants in vaccines also mediated their effects via activation of the inflammasome through GPRC6A. Again, the ablation of GPRC6A reduced IL-1β secretion in vitro and in vivo.[84] These studies, although limited in number, support a role of GPRC6A in inflammation.

2.8 | Summary of the proposed physiological functions of GPRC6A

Collectively, the present data on GPRC6A propose various physiological roles in multiple tissues (Table 1), which fit with the receptor's observed wide expression pattern and promiscuity with respect to ligands.

As evident from Table 1, there has been published several studies in the GPRC6A research field, which includes very different phenotypes for the different GPRC6A KO models. But why do the GPRC6A KO models display different phenotypes? This is a crucial question to answer in the future to enable the determination of the physiological function of GPRC6A and hence its clinical relevance and potential as a drug target.

2.9 | Putative causes to the observed discrepancies between knockout models

It is a well-characterized phenomenon that phenotypes differ from mouse strain to mouse strain as specific alleles in some strains can mask effects of some genetic mutations.[85] Hence, strain differences can constitute part of the explanation for the lack of consensus within the field. The group of Quarles generated the first GPRC6A KO model, which had a mixed C57BL/6 and 129X1/SvJ genetic background (white mice are depicted in the 2008 paper).[49] Our models have been backcrossed to the C57BL/6 genetic background, and it is therefore not ideal to compare results obtained using the exon VI and full locus models with data from the exon II model. Furthermore, it is hard to control a mixed genetic background, and abnormalities can be observed due to differences in genetic background rather than specifically due to the GPRC6A ablation.[86] Ideally, GPRC6A should be knocked out in multiple inbred and outbred strains to determine the function of the receptor, and to evaluate whether the C57BL/6 background masks the effects of losing GPRC6A.

TABLE 1 Overview of the main reported phenotypes for the three different GPRC6A knockout (KO) mice models. Not determined (ND), bone mineral density (BMD), wild-type (WT), L-arginine (L-Arg), glucagon-like peptide-1 (GLP-1)

Organ/tissue/system	Exon II KO	Exon VI KO	Full locus KO
Bone	↓BMD[49,73]	WT = KO[50]	WT = KO[56]
Metabolism	↑Basal glucose[49]	WT = KO[51]	WT = KO[53]
	↓Insulin serum levels[49]	WT = KO[51]	WT = KO[56]
	↓L-Arg-induced insulin secretion[47]	WT = KO[51]	ND
	Glucose intolerance[49]	WT = KO[51]	WT = KO[53]
	Insulin tolerant[49]	WT = KO[51]	WT = KO[56]
	↑Fat mass[49]	WT = KO[51]	WT = KO[56]
Inflammation	ND	↓Cytokines[83,84]	ND
Male fertility	↓Testosterone levels[27,49]	ND	ND
	↓KO-KO breeding[49]	WT = KO[a]	WT = KO[a]
Prostate cancer	Protected against prostate cancer[80]	ND	ND
Energy homoeostasis	↓Forced running (distance and time)[28]	WT = KO (speed)[61]	ND
	ND	↑Voluntary running[61]	ND
	ND	↑Diet-induced obesity[52]	ND
	ND	ND	Normal response to high-protein diet[53]
Nutrient-sensing	ND	Normal L-Arg-induced GLP-1 secretion[66]	Normal L-Arg-induced GLP-1 secretion[66]

[a]Unpublished observations.

In addition to the earlier mentioned potential differences in animal facility environments and underpowered studies,[54,55] another obvious explanation could be the GPRC6A targeting strategies. There could be sensitivity/resistance differences between the models depending on which exon that has been disrupted. Such an example has been published for the related CaSR, where different phenotypes were reported depending on the targeted region. When exon V encoding the extracellular domain was disrupted, a partial functional alternative splice variant was generated, which was able to compensate for the lack of full-length CaSR in tissues with high expression.[87] When exon VII, which encodes the 7TM domain, was deleted, a much more severe phenotype was observed.[88] A similar scenario for the exon II and exon VI GPRC6A KO models could explain the discrepancies. To avoid these possible exon-specific effects, we employed the full locus KO model in our latest studies. Overall, these studies did not demonstrate abnormalities of the full locus KO model, and thus, this model resembles the exon VI KO model with respect to glucose metabolism and bones.[56] It is therefore not likely that the exon VI model somehow can compensate for the ablation of the full-length GPRC6A receptor.

3 | CONCLUSION AND FUTURE PERSPECTIVES

Since the GPRC6A receptor was cloned in 2004, a number of studies have contributed with important knowledge on expression, ligands and signalling. In recent years, it has been demonstrated that the receptor forms homodimers through a disulphide bridge and that the receptor is constitutively internalized.[2,3] The physiological function of GPRC6A is still a mystery as in vivo mouse phenotyping studies have lacked consensus or remain unconfirmed by independent groups. The most recently obtained data using the full locus GPRC6A KO model have supported the lack of abnormalities of the exon VI KO model with respect to bones and metabolism and demonstrated that GPRC6A is not required for robust L-amino acid-induced GLP-1 release in vivo.[56,66] Other studies indicate a role of GPRC6A in male reproduction, prostate cancer and inflammation, and will be important to validate in other groups/mouse models before firm conclusions can be drawn.

We have recently revealed a SNP conferring a premature stop codon and a insertion/deletion in the third intracellular loop which causes intracellular retaining of

the human GPRC6A receptor.[7] Furthermore, hundreds of SNPs have been reported for the human *GPRC6A* gene.[8,9] This is an ideal starting point for working with human cohorts to reveal associations of GPRC6A and various phenotypes. This has the advantages of working with much greater population sizes than mouse studies and addressing human relevance directly. From there, the possible phenotypes can be tested in mice to delineate mechanisms and check if the mouse model holds translational value and subsequently GPRC6A can be evaluated as a potential drug target.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

CVJ and HB-O wrote the manuscript.

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